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13. ABSTRACT (Maximum 200) The cell surface hyaluronan receptor, CD44H, has been shown to promote progression of B16F10 murine melanoma in vivo, but the involvement of hyaluronan-CD44 interactions in this process remains unclear. Recently, we demonstrated <i>de novo</i> synthesis of soluble, secreted isoforms of CD44 via alternative splicing of novel exonic sequences. Since soluble CD44 would be expected to antagonize interaction between hyaluronan and membrane bound CD44, we investigated whether stable transfection of malignant tumor cells with cDNA encoding soluble CD44 would prevent their ability to form tumors in vivo. In the present study, we have used a murine mammary carcinoma line (TA3/St) which grows in ascites form and metastasizes through the peritoneal wall of syngeneic mice; these cells express CD44 and attach to regions of the peritoneal wall enriched in hyaluronan prior to invasion through the wall. We now find that when transfected TA3/St cells overexpressing soluble CD44 are injected intraperitoneally, they fail to attach and form widespread tumors in the peritoneal wall whereas wild type TA3/St cells or transfectants carrying vector alone form tumors rapidly and consistently. Also, unlike mice injected with wild type or mock-transfected cells, the mice injected with soluble CD44 transfectants do not accumulate ascites, indicating that tumor cell growth therein is also markedly reduced. These results together with our previous work imply strongly that soluble CD44 acts in this system as an antagonist of interactions between hyaluronan and membrane-bound CD44H that are essential for TA3/St tumor progression.				
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FOREWORD

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Antagonistic Action of Soluble CD44 and Hyaluronan Oligomers in Breast Cancer

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5) Introduction

Hyaluronan Influences Cell Behavior

Interactions between cells and their surrounding extracellular matrix are known to influence growth, migration, adhesion, and apoptosis of various cell types. Of particular importance is the role of matrix in stabilizing the differentiated state of cells. Subtle alterations in normal matrix properties can lead to aberrant cell division, escape from normal barriers to cell movement, and even cell death. One of the macromolecules commonly found in extracellular matrices is the polysaccharide, hyaluronan. Hyaluronan (HA) influences migration, growth and adhesion of cells through interaction with cell surface hyaluronan receptors (1-3). It is now known that in some tumor types the interactions of hyaluronan with these receptors is an essential component of the broad cascade of events necessary for tumor growth and metastasis (4-7), although investigation of the steps leading from hyaluronan-receptor interaction to intracellular alterations that induce aberrant cell behavior is still in its infancy. The influence of hyaluronan on cell behavior, and the mediation of these events by cell surface hyaluronan-binding proteins (HABPs), has long been the focus of investigations going on in our laboratory. These studies, as well as the results obtained in other laboratories, have demonstrated that the interaction of HA with many different cell types is likely to be fundamental to several aspects of cell behavior during embryonic development, tissue regeneration, and tumorigenesis (1-7).

Investigations into the effects of HA on cellular processes have shown: a) that HA is enriched in matrices in which cells migrate and proliferate *in vivo*; and, b) that HA influences cell behavior *in vitro* and *in vivo* (1-7). For example, HA stimulates movement of several cell types (10,13). Additionally, the inclusion of HA promotes cellular invasion of collagen gels, whereas the inhibition of the HA-HABP interaction, or the removal of endogenous HA by hyaluronidase treatment, blocks such invasion (14). HA has also been shown to mediate cell adhesion in several cell types (15,16), which may be vital to some aspects of embryonic development (17), tumor invasion and metastasis (18), and the immune response (19,20).

Cell Surface HABPs Mediate the Effects of Hyaluronan

Characterization of cell surface HABPs (HA receptors) that mediate HA-cell interactions is an area that has undergone dramatic expansion over the past several years. The best characterized HA receptors are CD44 and RHAMM. Several other HABPs have been identified but only RHAMM and CD44 have been convincingly shown to mediate cellular effects of HA. The CD44 gene produces an incredibly diverse group of proteins. At least 10 variant exons are alternatively spliced in various combinations into the extracellular region of the CD44 gene, and approximately 20 isoforms from various tissues and cell types have been isolated thus far (21,22). In addition, CD44 is posttranslationally modified by O- and N-glycosylation, glycosaminoglycan addition, and phosphorylation, all of which influence the reactivity of CD44 (23-25). The most predominant CD44 isoform is CD44H, and hyaluronan-CD44H interactions are thought to mediate endocytosis of hyaluronan (26,27) as well as various aspects of cell aggregation (16), cell adhesion (15,16), pericellular matrix assembly (8,9), and cell migration (10). CD44H acts as a major transmembrane HA receptor (28), and it is widely distributed in several tissue types. This standard form (CD44H), as well as all isoforms of CD44, include the HA-binding domain in their N-terminal regions despite their varying capacity to bind hyaluronan (21). Although the physiological functions of variant CD44 isoforms as HABPs remain unclear (3,20), they are mainly restricted to tumor cells, activated lymphocytes, and proliferating or morphogenetically active epithelia (22,29,30). Putative ligands for CD44 variants other than HA most likely exist, but their identities and functions are not well established.

Since cell surface HA-HABP interactions initiate many cellular effects of HA, the biochemical mechanisms by which these interactions are transduced into intracellular signals that bring about these effects are now being intensely studied by several groups. HA-CD44 interaction

causes an increase in intracellular Ca^{++} , clustering of CD44 in the membrane and accumulation of ankyrin beneath it (31,32,33). CD44 interacts with the tyrosine kinase, p56^{lc} , leading to increased phosphorylation of ZAP-70 and other intracellular proteins (34). CD44 also interacts with proteins associated with the cytoskeleton (31,35,36) and with lipids in the plasma membrane (37). Glycosylation of the extracellular domain (24) and integrity of the cytoplasmic tail (33,35,36,38) greatly influence HA binding. RHAMM also associates with cytoskeletal elements (39) and triggers a series of intracellular signaling events (40).

HA-HABP Interactions Modulate Cell Behavior During Tumorigenesis

The study of tumorigenesis is the most developed area of investigation implicating HA-HABP interactions in vivo. Several studies have shown: a) that invasive tumors produce elevated levels of HA in vivo compared to their benign counterparts (5,41); b) that direct and indirect interactions between tumor cells and stromal cells stimulate HA production by the latter (5,42,43); and c) that some metastatic tumor cells have much higher levels of cell surface HABP than their benign counterparts (44-46). It has recently become clear that CD44 and RHAMM are both involved in transformed and tumor cell migration, and various isoforms of CD44 and RHAMM are involved in tumor growth and metastasis. It has also become apparent that HA-CD44 and HA-RHAMM interactions lead to intracellular signaling events that modify cell behavior (31,47). Of particular interest is a recent study in which it was shown that HA initiates locomotion of ras-transformed fibroblasts via RHAMM-induced tyrosine kinase activity (47), and ultimately leads to metastatic behavior in vivo (6). A very rapid response to HA-RHAMM interaction is transient phosphorylation of p125^{FAK} in concert with turnover of focal adhesions (47). These events do not occur if HA-RHAMM interaction is suppressed, leading to stabilization of focal contacts and loss of motility (6). Transfection of CD44-negative lymphoma cells with cDNA for CD44H promotes tumor growth and metastasis (48). Administration of either antibodies that block HA binding to CD44H (49), soluble CD44-Ig fusion constructs that compete for HA-CD44H interaction (7), or HA oligomers that block HA-HABP interaction inhibits melanoma growth and/or metastasis (11). Furthermore, introduction of a single base mutation in CD44H that interferes with HA binding nullifies its efficacy in vivo (7). With respect to signaling, it appears likely that polymeric HA induces clustering of CD44 receptors (50) which may in turn be important for signal transduction, leading to modified cell behavior (31). Clustering of CD44 splice variants in rat carcinoma cells increases the binding affinity of soluble HA for CD44, thereby suggesting that HA binding is dependent of the ability of CD44 to aggregate in the plasma membrane (50). The loss of cell surface CD44 in colon carcinoma cells observed during CD95-induced apoptosis is also proposed as one of the first steps that may contribute to detachment of adherent cells during programmed cell death (51). Even though it can be seen that both CD44 and RHAMM play important roles in many aspects of tumor growth and progression, the focus of our project centers on HA-CD44 interactions during tumorigenesis.

HA oligomers and Soluble CD44 Block Tumor Growth

Past studies in our laboratory have shown that oligosaccharides of hyaluronan, as small as hexamers, competitively inhibit interaction of hyaluronan polymer with receptor. These oligomers also block migration of, and pericellular matrix assembly by, some embryonic cell types (8-10). Of particular interest is the finding that they inhibit formation of murine melanomas in vivo (11). The physiological action of hyaluronan polymer is likely to be mediated through clustering of its receptors, brought about by multivalent interaction of oligomeric sequences with multiple receptor units. Receptor clustering would then result in transmission of intracellular signals as happens with many other signal transducing, bi- or multi-valent, receptor-ligand interactions. We propose that hyaluronan oligomers act as antagonists that block hyaluronan polymer binding and resultant signal transduction. The latter would occur since the oligomers are too small to span several receptor units and thus would not induce the receptor clustering that may be necessary for signal

transduction. In addition, soluble CD44-Ig fusion proteins (7,10,13) and anti-CD44 antibodies (52,53) have also been shown to block progression of melanomas, lymphomas and ovarian carcinomas. Possible explanations of these findings could be that molecules that block the polymeric HA-HABP interaction, such as soluble CD44 and HA oligosaccharides, may interfere with this receptor clustering and resultant signal transduction. Our group has recently identified CD44 isoforms that are secreted due to lack of membrane and cytoplasmic domains (12), that may also have the capacity to act as antagonists of membrane CD44H (7,10,13). A newly described exon can be alternately spliced to create transcripts containing stop codons, thereby providing a molecular basis for *de novo* synthesis of a soluble CD44 variant. As with several other adhesion molecules, soluble CD44 has been detected in substantial amounts in serum, lymph, and synovial fluid from a variety of species (54). In addition, elevated levels of soluble CD44 have been detected in the sera of tumor patients (55-57). We hypothesize that antagonists to either membrane bound CD44H, such as soluble CD44, or to hyaluronan polymer, such as HA oligosaccharides, inhibit HA-CD44 interactions that are crucial to the cellular processes involved in breast cancer tumorigenesis (Figure 1). Consequently, we propose to investigate the inhibition of mammary

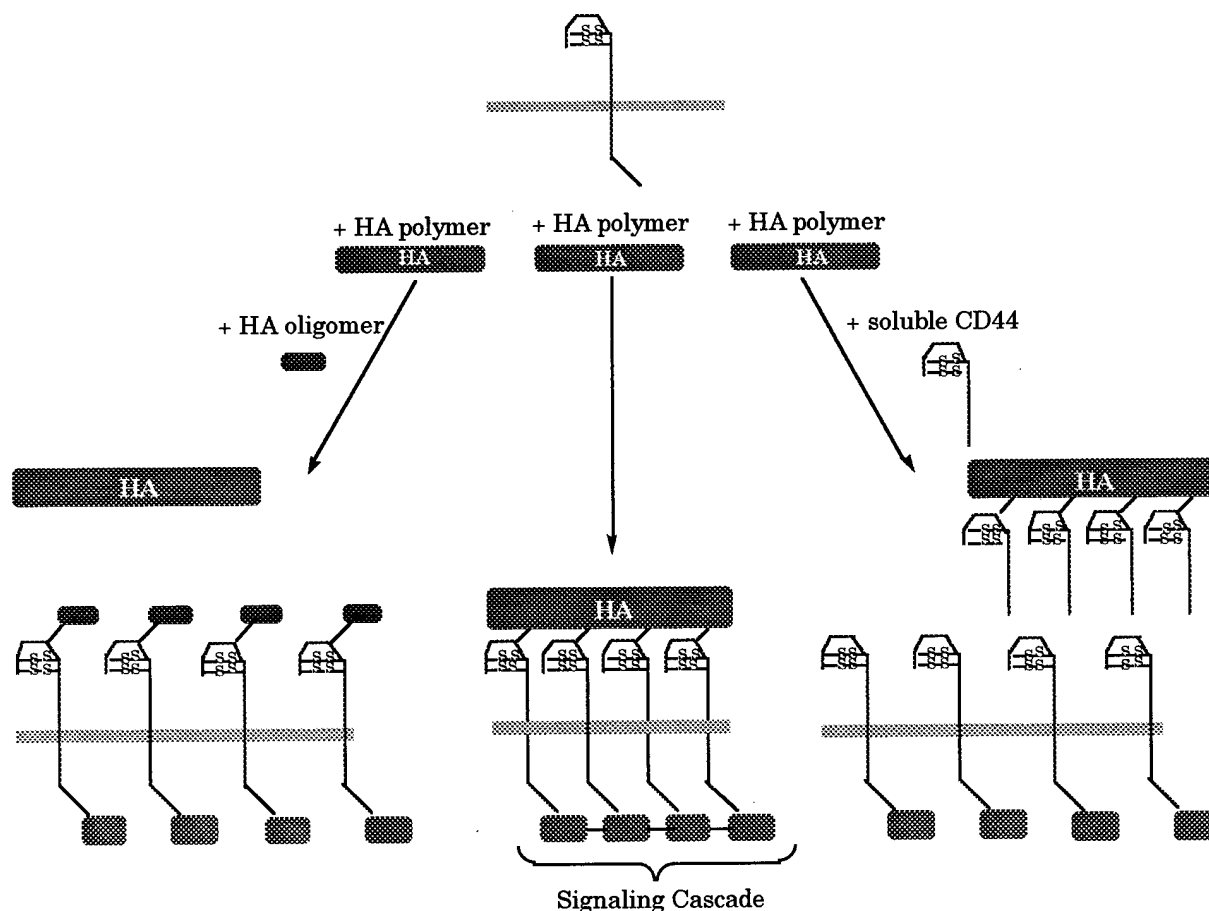


Figure 1: A model for the inhibition of hyaluronan-CD44 interactions by HA oligomers and soluble CD44.

tumor progression by soluble CD44 and HA oligomers in vivo. This report will focus on the effect of overexpression of soluble CD44 by transfection of tumor cells on murine mammary carcinoma growth and invasion in vivo within an intraperitoneal mouse model system.

Attachment Sites of CD44-Positive Tumor Cells Show Increased HA levels

Previous studies in our laboratory by Dr. Tet-Kin Yeo have investigated murine breast (TA3/St) and ovarian (MOT) ascites tumors as models to follow changes in HA levels during tumor growth, attachment and invasion in vivo. Ovarian and breast cancer cells frequently exfoliate into body cavities where they induce accumulation of ascites fluid to enable the tumor cells grow in suspension. A portion of these cells have been shown to attach and grow on mesenteric surfaces and consequently, especially in the case of breast cancer, invade these tissues. Subsequent to introduction of tumor cells into the peritoneal cavity, the amount of HA in the ascites rises from an undetectable level to peak values of 45-200 mg/l at 5 to 9 days post-injection. Using a specific probe to visualize HA, local HA accumulation was observed at initial sites of attachment of tumor cells to the surface mesentery (18). However, the tumor cell lines synthesize very low amounts of HA in culture. Therefore, it was concluded that the observed HA accumulation in vivo results from increased synthesis and secretion by mesothelial cells and/or fibroblasts, most likely in response to stimulation by direct interaction with the surface of tumor cells (42) or by their secreted products (43).

Virtually all of the TA3/St tumor cells that initially attach to mesentery are strongly positive for CD44. At later stages both CD44-positive and negative cells accumulate within the peritoneal wall. Following attachment, the TA3/St mammary tumor cells invade the mesentery and HA is deposited at the tumor-host interface. Thus, it was proposed that an HA-rich matrix promotes tumor cell attachment and invasion by interaction with tumor cell surface CD44 (18). As mentioned previously, this is supported by studies demonstrating inhibition of attachment of ovarian carcinoma cells to mesothelium in vitro and in vivo by antibody to CD44 (52,53) and inhibition of melanoma progression in vivo by soluble CD44-Ig fusion constructs (7). Therefore, we decided to further test the mediation of murine mammary (TA3/St) tumorigenesis by HA-CD44 interactions between the tumor cells and the surrounding HA-rich matrix found in this model system.

6) Body

Experimental Methods

Cells and cell culture TA3/St tumor cell lines transfected with soluble CD44 or vector alone were maintained in DMEM containing 10% fetal bovine serum and 500 μ g/ml G418 sulfate.

Animals Syngeneic female A/Jax mice were injected intraperitoneally with 1×10^6 transfected or wild-type tumor cells suspended in 200 μ l Hanks' balanced salt solution (HBSS). The mice were then monitored for ascites accumulation and sacrificed at either 7 days or >14 days post injection.

Histology After mice injected with TA3/St cells (transfectants and controls) were sacrificed at each of the two time points (7 to >14 days post injection), the peritoneal walls of these mice were harvested, cut into strips approximately 5mm x 15mm and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for approximately 12 hours. Subsequently, the tissues were washed in PBS, dehydrated through a series of ethanol (30%, 70%, 95%, 100%) and xylene washes and embedded lengthwise in paraffin wax blocks to ensure that the cut sections would be cross-sections of the mesothelium and the muscles of the peritoneal wall. The blocks were cut into 5 μ m sections, mounted on slides and stained with hemotoxylin and eosin.

Results and Discussion

Inhibition of Ascites Tumorigenesis in vivo by transfection with cDNA for Soluble CD44

As mentioned previously, our group has identified CD44 isoforms that are secreted due to lack of membrane and cytoplasmic domains (12). These soluble isoforms arise because of stop codons found in three newly discovered inserts, or exonic sequences, that can be alternatively spliced to create these soluble forms of the CD44 protein (Figure 2a). Since soluble CD44 would be expected to antagonize the interaction between hyaluronan and membrane bound CD44H, we investigated whether stable transfection of malignant tumor cells with cDNA encoding one of these soluble CD44 isoforms (Figure 2b) would prevent their ability to form tumors in vivo. We used the above-described murine mammary carcinoma line (TA3/St) which grows in ascites form and metastasizes through the peritoneal wall of syngeneic mice. As stated above, studies in our lab have previously shown that these cells express CD44 and attach to regions of the peritoneal wall enriched in hyaluronan prior to invasion through the wall (18).

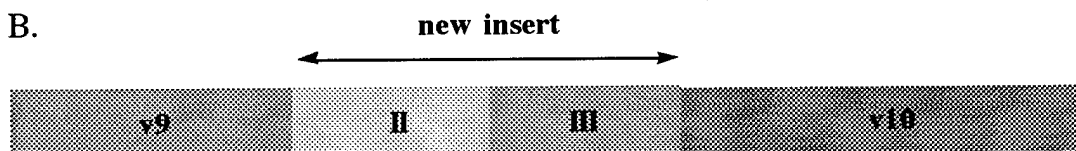
Stable transfectants were analyzed by PCR and Western blotting techniques to confirm that each clone carried the cDNA of the vector and the soluble CD44 gene or that of vector alone, as well as to show that the protein was indeed being produced and secreted. The transfectants were then grown in media enriched with G418 sulfate that would ensure the selection of subsequent generations in culture that carry the *neo* vector cDNA. Transfected TA3/St cells overexpressing soluble CD44 (63A, 63B) were injected intraperitoneally into syngeneic female A/Jax mice and allowed to grow within the peritoneal cavity of these mice for 7 to >14 days post injection. In parallel control experiments, wild type TA3/St cells and TA3/St cells transfected with vector alone (Controls 1 and 8) were also injected in the same concentrations. We evaluated several animals in each of the above conditions by determining the degree of ascites growth and TA3/St cell

A.

```

v9  GTAAGGATCATAAAGTCCAACTGGCTTTTAAGCAGAAATCAAGACGTTAT
      GGGTGTGTCTGGTGGTGGTTGCTGATTTTCTGCTTTATAGATCTTTTAGAA
      CCATTGTACACTAGGTACAACTTTTCTGCTGAATTTTAAAG v10
  
```

B.



Amino acid sequence of new insert: VCLVVVADFSAL***

Figure 2: **Sequence and arrangement of new insert between v9 and v10.**

A, Various combinations of three novel inserts give rise to soluble isoforms of CD44. These inserts are designated: I, II, and III. Stop codons that result from these inserts are marked with triple asterisks (***); the particular stop codon introduced depends on the combination of inserts used [Yu and Toole, (1996) *J. Biol. Chem.* 271: 20603-20607]. B, The cDNA used for producing stable transfectants contained the variant exons, V6, V7, V8, V9 and inserts II and III. Two separate clones obtained from the transfection were used: 63A and 63B.

attachment and invasion into the peritoneal wall, and the results are summarized in Table 1. We found that TA3/St cells overexpressing soluble CD44 usually failed to form tumors in the peritoneal wall or mesentery whereas wild type TA3/St cells or transfectants carrying vector alone formed tumors rapidly and consistently. At 7 days post-injection, tumor cells in animals injected with control (vector alone) transfectants or wild-type TA3/St cells can be seen to line up along the mesothelium as they attach to the inside of the peritoneal wall (Figure 3a,3b). Yet, in animals injected with transfectants overexpressing soluble CD44 (Figure 3c,3d), the mesothelium remains relatively smooth and unchanged. Likewise, after a period exceeding 14 days post-injection, mice carrying TA3/St transfectants overexpressing soluble CD44 are also seen to have peritoneal walls that appear generally unaffected (Figure 3g,3h)) when compared to the peritoneal walls of mice injected with control transfectants or wild-type cells which are seen to have widespread tumor cell attachment and invasion into the peritoneum (Figure 3e,3f). Yet, it should be noted that transfectants were found to attach to a limited extent to the peritoneal wall of three out of 12 mice sacrificed at >14 days post-injection. However, the transfectants attached at a few discrete foci only, whereas the controls formed continuous masses of tumor cells along and within the mesothelium and peritoneal wall. In these three cases small tumors were also observed, but they were isolated and surrounded by intact mesothelium very unlike the tumors seen in the tissues from control animals that exhibited widespread and uniform invasion throughout the peritoneal wall. In addition, unlike mice injected with wild type or mock-transfected cells (Figure 4a), the mice injected with soluble CD44 transfectants do not accumulate ascites (Figure 4b), indicating that tumor cell growth therein is also markedly reduced. Therefore, together with our previous work, these results imply strongly that soluble CD44 acts in this system as an antagonist of interactions between hyaluronan and membrane-bound CD44H that are essential for TA3/St tumor progression.

Table 1:

Reversal of Tumorigenicity in TA3/St Mammary Carcinoma Transfectants					
Cell Type	Time Elapsed Before Analysis	# of Mice	Attachment at 7 days	Invasion >14 days*	Ascites Growth
Controls:					
TA3/St w/t	7 days	6	6	na	na
	>14 days*	6	na	6	6
TA3/St vector alone	7 days	6	6	na	na
	>14 days*	6	na	6	6
Transfectants:					
TA3/St 63A	7 days	6	0	na	na
	>14 days*	6	na	1 [#]	0
TA3/St 63B	7 days	6	0	na	na
	>14 days*	6	na	2 [#]	0

* Animals were examined between 14 and 19 days after injection.

[#] Limited and inconsistent attachment; small tumors or foci regions of tumor cell accumulation.

na: Not applicable for the mice sacrificed at this time point.

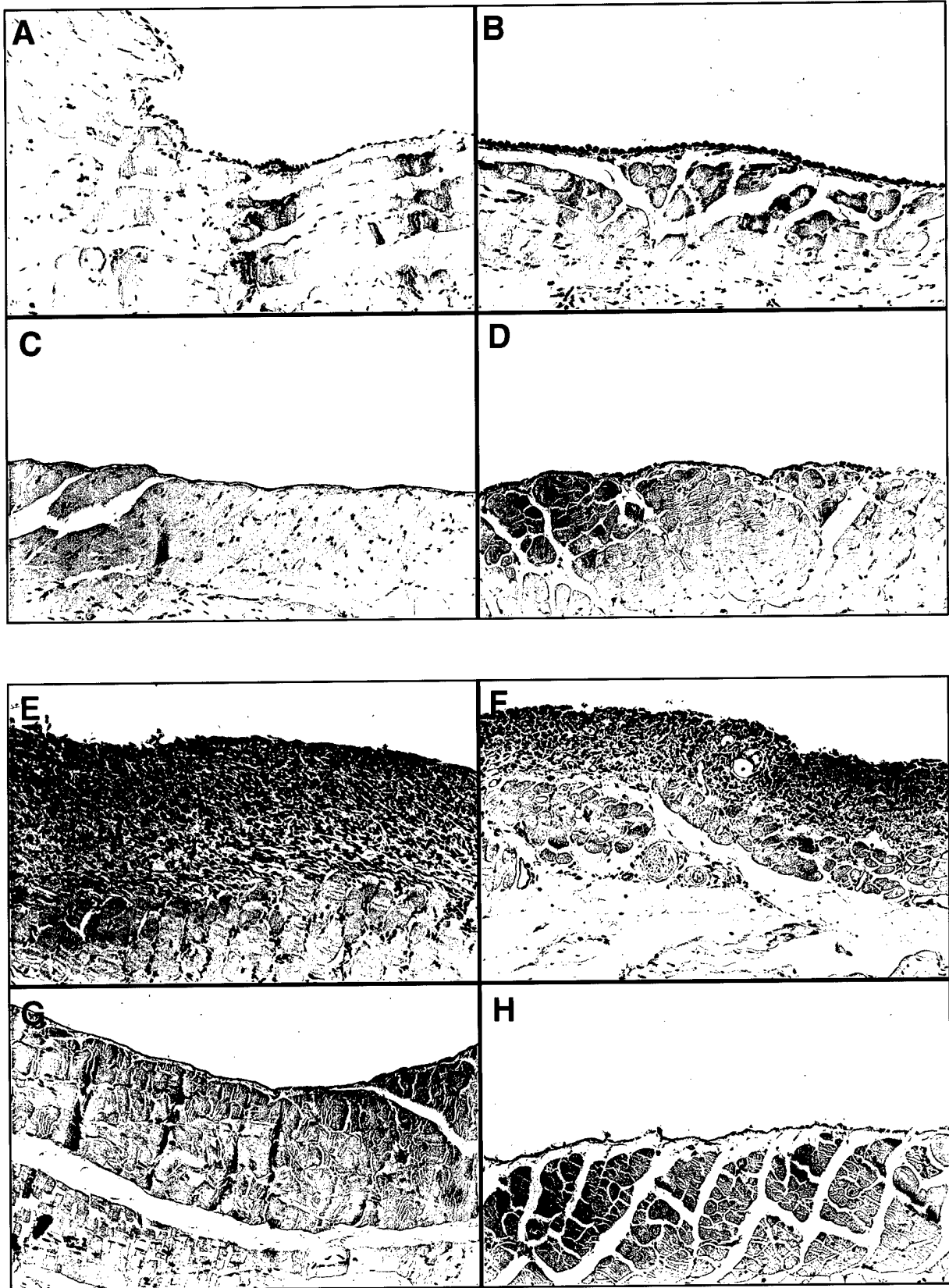


Figure 3. Reversal of tumorigenicity in TA3/St transfectants. TA3/St transfectants over-expressing soluble CD44, mock transfectants or wild type TA3/St murine mammary carcinoma cells were injected into the peritoneal cavity of syngeneic mice. After 7 (A-D) or >14 days (E-H) the mice were sacrificed and then the peritoneal wall was fixed and stained. A and E, wild type TA3/St. B and F, mock transfectant C1. C and G, transfectant 63A. D and H, transfectant 63B.

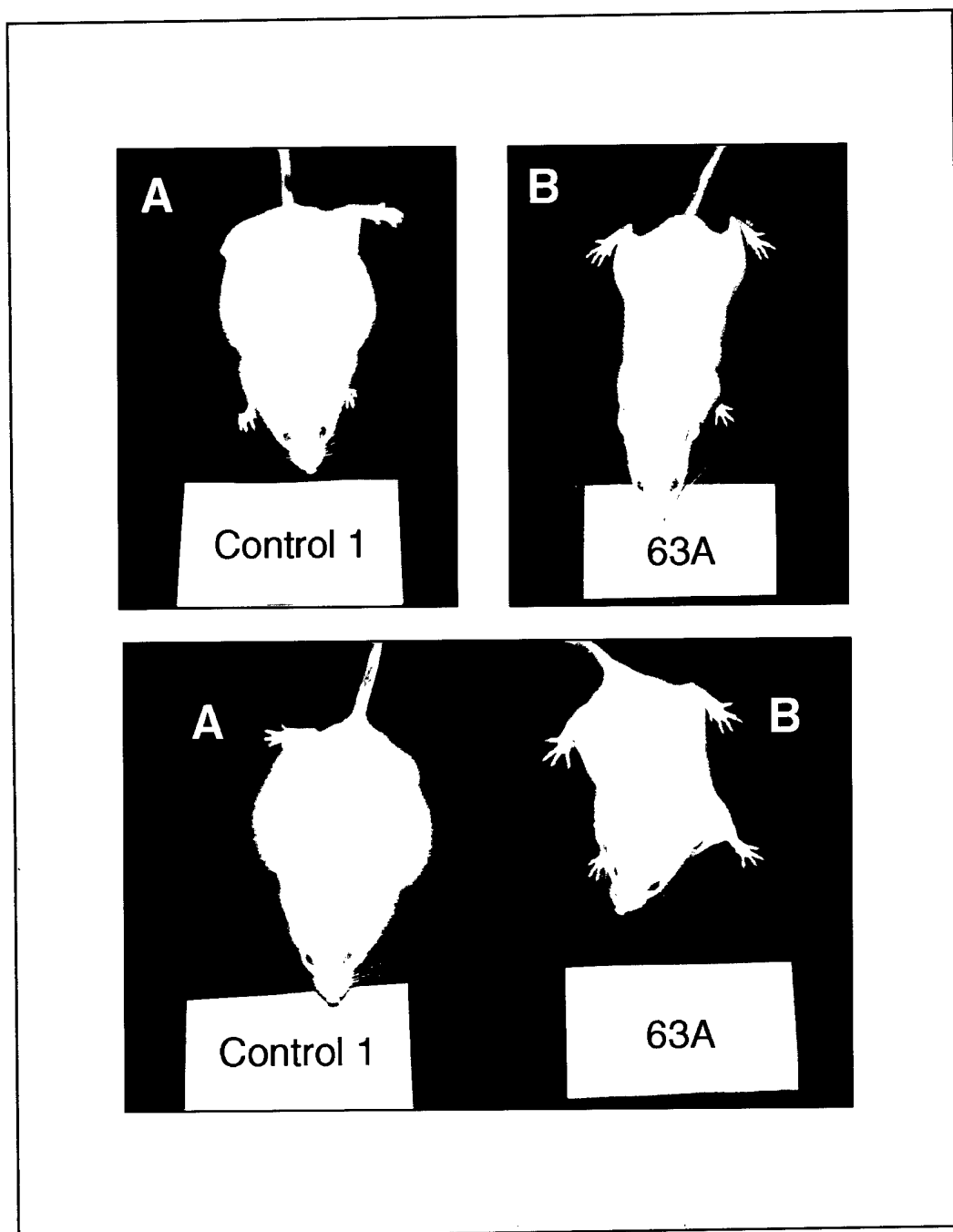


Figure 4. Mice injected with transfectants overexpressing soluble CD44 do not accumulate ascites fluid. A, control mouse injected with mock-transfected TA3/St cells and photographed after 18 days. B, mouse injected with transfectant, 63A, and photographed after 18 days. The abdominal swelling in the control mouse is due to accumulation of ascites fluid caused by growth of the TA3/St tumor cells in the peritoneum. This swelling is not seen in the mouse injected with transfectant TA3/St-63A.

7) Conclusions

HA-HABP interactions have been implicated in several different investigations of tumorigenesis *in vivo*. Various studies have demonstrated that HA levels are elevated *in vivo* by highly invasive tumors (5,18,41), and that some metastatic tumors have more HABPs than their benign counterparts (44-46). Furthermore, antagonists to the HA-HABP interaction, especially in the case of HA-CD44 interaction, have been shown to abate tumor progression both *in vitro* and *in vivo*. For example, soluble CD44-Ig fusion proteins (7), and HA oligosaccharides (11), have been shown to block the progression of melanomas. In addition, the administration of antibodies against CD44 significantly reduced the number of ovarian cancer implants within a nude mouse xenograft intraperitoneal model (53) that is similar to the system employed in our studies. Since both ovarian and breast cancer cells often exfoliate into body cavities where they induce the accumulation of ascites fluid necessary for their survival and growth, an intraperitoneal model is an excellent environment for studying the tumorigenicity and metastatic potential of breast carcinomas. Therefore, by evaluating the behavior of these cells in this animal system, we have been able to gain insight into which extracellular matrix components may be contributing to the highly metastatic and lethal nature of these cells.

In this study we have demonstrated that the transfection of a TA3/St breast carcinoma cell line with cDNA for soluble CD44 inhibits the tumorigenicity of these cells in a syngeneic murine intraperitoneal model system. Previous studies in our laboratory have shown that the introduction of TA3/St tumor cells into this mouse model has resulted in the dramatic accumulation of hyaluronan in ascites fluid and on the surface mesentery at initial attachment sites of the tumor cells (18). Furthermore, these studies also demonstrated that almost all of the TA3/St tumor cells that initially attach to the mesentery are strongly positive for CD44, and that this tumor-host interface is enriched in HA. Therefore, we hypothesized that this HA-rich matrix surrounding the tumor cell attachment sites may promote the cell attachment and invasion into the host tissues, and we proposed that the disruption of the interaction between the HA on this interface and the CD44 receptors of the tumor cells may inhibit the tumorigenicity of these cells by prohibiting their attachment and invasion into the mesothelium within the peritoneal cavity. Through the overexpression of soluble CD44 in the TA3/St cells, we have shown that the tumorigenicity of these cells is markedly reduced (Table 1). This suggests that soluble CD44 may be acting as a sink for endogenous HA within the peritoneal cavity, thereby inhibiting the interaction between the CD44 on the surface of the TA3/St cells and the endogenous HA that has become bound to the soluble CD44 secreted from these cells.

In summary, our results provide more evidence that the CD44 receptor is intimately involved in the attachment and invasion of breast cancer cells *in vivo*, and that the inhibition of the CD44-HA interaction by soluble CD44, or other potential antagonists, may be a useful strategy for reducing the spread of this disease. Since hyaluronan-receptor interactions are essential in many cases of tumor progression, soluble CD44 may be especially useful for treatment of breast cancer cases, where self examination and other diagnoses often reveal mammary tumors prior to metastasis. In some of these cases HA-HABP antagonists, like soluble CD44, could be administered locally subsequent to surgery to prevent further tumor progression and invasion into the host tissues. Therefore, the continuing goal of our research will be to focus on identifying other antagonists, such as hyaluronan oligosaccharides, that effectively interrupt the HA-CD44 interaction and significantly limit tumor progression and metastasis of breast carcinomas. Further studies will hopefully yield an HA-HABP antagonist that optimally inhibits tumorigenesis as well as abates tumor growth of existing malignancies.

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